

Do morphine and its metabolites morphine-6-glucuronide and morphine-3-glucuronide affect dopamine release in nucleus accumbens in mice?

André Gottås

Master thesis

University of Oslo, Norway

Department of Biology, Faculty of Mathematics and Natural Sciences,

February 2009

Acknowledgements

This Master of Science thesis has been carried out at the Norwegian Institute of Public health, Division of Forensic Toxicology and Drug Abuse (Oslo, Norway), in the period between 2007 and 2009.

I will first thank my supervisor, Fernando Boix, for the opportunity to participate in such an interesting field of research, and for his support throughout the project. Also to Steinar Øvrebø for his assistance when needed.

I will also thank Vigdis Vindenes for excellent support with the laboratory experiment, and for being there when questions were raised.

Björg Sjøgren Pettersen deserves thanks for the assistance with sample analysis, her experience and effort was indispensable when troubleshooting was required.

Nina Lieng did a mayor job doing all the slicing and colouring of “my little brains”, so I could focus on performing the examination of probe placement. I am grateful for your support.

I also want to thank Åse Ripel for different laboratory help.

My fellow students, you have made this past five years memorable! Harald, my comrade from the great city of Halden and fellow “tox” student, thanks for the fun times, great squash sets, and constructive brainstorming sessions. The rest of the gang in reading room 4131, each visit has been a joy because of you! To all my bio babes, what a tremendous pack of girls you are, I have been enjoying every minute in your company! To my potential wife out there, thanks for being sporty waiting up for me while I’ve been busy getting smart... Vegard and Arild, it has been great having some fellow guys around. Thanks for your advices when writing this thesis, Vegard!

I would like to take the opportunity to exalt FK Skruknott, Oslo’s finest football club, which is responsible for me not being obese, just stout. Looking forward for the matches and practices with these great friends has been vital in tough times.

Last but not least, mom and dad, your “creation” is finally done with studying. You have always made sure that I’m well, supporting me in all possible kind of ways. Coming home on the weekends has been, and will always be, the best thing! To the rest of my family and friends who have been there and encouraged me underway, you all mean a lot to me!

Abstract

Opiates have been used for centuries both as recreational drugs and for medical purposes. Opioids are substances that bind opioid receptors that are present throughout the nervous system. These receptors are the primary mediators of the pharmacological effects of opioids. However, their addiction potential is due to their rewarding/reinforcing effects conveyed by activation of dopaminergic neurons in the ventral tegmental area (VTA). Activation of VTA neurons leads to an increased release of dopamine in the nucleus accumbens (NAc), which is an important mechanism related to the reward/reinforcing effect of a variety of drugs and natural stimuli.

Heroin is rapidly metabolized to 6-monoacetylmorphine after intake, which is further converted to morphine. Morphine is metabolized to morphine-6-glucuronide (M6G) and morphine-3-glucuronide (M3G). M6G has recently been showed to have rewarding effects similar to morphine, while M3G has not showed such effects. It is well known that morphine, has rewarding effects and increase dopamine release in the NAc. The effects of the morphine metabolites on mesocorticolimbic dopamine release have not yet been studied. The present study thus aims to investigate how morphine, M6G and M3G affect extracellular concentrations of dopamine in the NAc by using the in vivo microdialysis technique in freely moving mice. Microdialysis samples were analyzed for dopamine by high performance liquid chromatography (HPLC) with electrochemical detection (ED) after injection of different doses of morphine, M6G and M3G.

Both morphine and M6G treatment significantly increase extracellular dopamine concentrations in NAc, but a dose-response relationship was not seen for the two doses used. Administration of M3G shows no significant increase in extracellular dopamine. This study therefore confirm previous results indicating that M6G, but not M3G, have addictive properties and thus might play an important role in development of dependence after heroin or morphine administration.

Table of contents

1. Introduction	3
1.1. Opiates	3
1.1.1.1. Pharmacology of opiates	3
1.1.1.2. Opioid receptors	4
1.1.1.3. Pharmacological effects of opioids	5
1.2. The nervous system underlying opioid addiction	8
1.2.1. The mesocorticolimbic dopamine system	8
1.2.2. Dopamine and dopamine receptors	10
1.2.3. Learning, reward and addiction	11
1.3. The microdialysis technique	12
1.4. Aim of the study	12
2. Materials and methods	14
2.1. Animals and conditions	14
2.2. Surgical procedures	14
2.3. In vivo microdialysis experiment	16
2.4. Experimental protocol	17
2.5. Chromatographic analysis of brain samples	18
2.6. Histological verification of probe localization	18
2.7. Drugs	19
2.8. Data analysis	20
3. Results	21
3.1. Dopamine concentrations	21
3.2. Histological verification of probe localization	24
4. Discussion	25
4.1. Purpose	25
4.2. Choice of method	25
4.3. Opiate metabolites and dopamine release	25
4.4. Methodological challenges	27
5. Conclusion.....	30
6. References	31

1. Introduction

1.1 Opiates

Opiates are traditionally extracted from the Opium poppy (*Papaver somniferum*) or produced in laboratories as synthetic opioids. Morphine and codeine are examples of natural opiates, whereas naloxone and heroin (diacetylmorphine) are semi synthetic opioids produced from opium, and methadone is an example of a fully synthetic opioid product.

Opiates have been used for centuries in folk medicine to cure pain, diarrhea, cough and other ailments, as well as in religious rituals and as recreational drugs. In modern medicine, opioids are mainly used as pain relievers. In either case, used as medicine or recreational drug, addiction to opiates is a major problem for the individual as well as the society. Addiction to opioids might lead to social problems e.g. criminality, prostitution, diseases, injuries, accidents etc. This negative social and individual consequence generates high resource costs on both levels. An economic estimate done in the United States in 1996 concluded with a total cost of heroin addiction at 21.9 billion US\$; from them, 5.2 billion US\$ was due to criminal activity, 11.5 billion US\$ caused by lost productivity, and another 5.0 billion US\$ was due to medical care costs (Mark *et al.* 2001). Chronic opioid addiction is still difficult to treat, and the relapse percentage is relative high after detoxification and treatment. Therefore, it is important to increase our knowledge on the neurological mechanisms that induce and maintain opioid addiction, in order to promote the development of more effective treatments.

1.1.1 Pharmacology of opiates

Morphine has been the most used analgesic in modern medicine for decades. When administrated to relieve pain, the analgesic effect is prominent. Morphine is also used as a recreational drug, with intoxication and hedonia/ euphoria as the more marked effects. The pharmacological effect of heroin is also believed to be mediated trough morphine, as one of its major metabolites. Heroin is rapidly deacetylated to 6-monoacetylmorphine (6MAM) by carboxylesterase-1, carboxylesterase-2 and pseudocholinesterase (CE-1, CE-2 and pseudo-ChE) bound to the surface of red blood cells among others (Aderjan & Skopp 1997). These same enzymes convert further 6MAM to morphine (Maurer *et al.* 2006; Rook *et al.* 2006). The half life of heroin in human blood is on an average 1.3-7.8 minutes (Rook *et al.* 2006).

Such short half life, besides its poor receptor efficacy (Inturrisi *et al.* 1983; Rook *et al.* 2006), indicates that heroin itself does not exert significant pharmacological effects, thus being considered as a prodrug exerting its effect through its metabolites. When heroin is used clinically or for intoxication purpose, it is preferably administered parenteral, avoiding the complete first-pass metabolism by hepatic and extra hepatic factors. Heroin has a higher degree of lipophilicity compared to morphine that leads to a more effective penetration through the blood brain barrier (BBB), thereby delivering a more intense and immediate pharmacological effect (Oldendor *et al.* 1972; Inturrisi *et al.* 1984). The degree of involvement of different metabolic pathways depends largely on the route of administration, i.e. parenteral or orally. Morphine, either directly administered or formed after metabolism of heroin, is further metabolized to morphine-6-glucuronide (M6G) and morphine-3-glucuronide (M3G) by uridine diphosphate glucuronyltransferases (UGTs), where UGT2B7 is the major contributor (Coffman *et al.* 1997; Lotsch 2005; Maurer *et al.* 2006). Morphine is also, but in a lesser extent, N-demethylated by hepatic CYP3A4 and CYP2C8 to normorphine, or conjugated with sulphate, catalyzed by sulphotransferase or sulphokinase, to morphine-3-sulphate (M3S) and morphine-6-sulphate (M6S) (Maurer *et al.* 2006). These metabolites may also further be metabolized through glucuronidation in the same manner as morphine. The glucuronated or sulphated morphine compounds are highly hydrophilic, so the main excretion is via urine with a minor amount through bile. In humans, the main excretion metabolite is M3G, accounting for 50-60 % of excretion, M6G and morphine representing 8-10 % and 5-10 % respectively. M3S stands for 5-10 %, and normorphine for 1-6 % excretion, including their glucuronated conjugates (Aderjan & Skopp 1997; Rook *et al.* 2006).

1.1.2 Opioid receptors

Opioid receptors have multiple functionalities and are distributed throughout the nervous system, both in the central nervous system (CNS) and peripheral nervous system (PNS). Opioid receptors are involved in nociception as well as in reward, but also emotional, autonomic, neuroendocrine and immunological responses are affected (Kreek *et al.* 2002; Kieffer & Evans 2009). Based on its amino acid sequence, the opioid receptors are members of a larger family of receptor proteins, namely the rhodopsin-like G-proteins. Other members of this family are receptors for many peptide neurotransmitters, receptors for the monoamine neurotransmitters dopamine, serotonin, adrenaline and noradrenaline, as well as for acetylcholine (Kieffer & Evans 2009). The opioid binding sites are within the seven

transmembrane helical structure of the receptor protein, which has a relative high homology between the different receptor subtypes. The current hypothesis for selectivity for agonist or antagonist ligands is that the extracellular N-terminal domains, which are less homologous, are functioning as gates that allow entering of their respective ligands (Kieffer & Evans 2009). Endogenous ligands for opioid receptors are the opioid peptides, like endorphins, endomorphins, enkephalins and dynorphins. The receptors are divided into three subtypes related to their N-terminal domain and named with the prefix μ (mu), δ (delta), or κ (kappa) opioid receptors (also known as MOP, DOP and KOP receptors respectively)(Kreek *et al.* 2002; Kieffer & Evans 2009). Opioid receptors are G-protein coupled and the effect of ligand binding and activation can initiate different intracellular responses depending on the G-protein in function: the G_i subtype protein inhibits the cAMP production pathway, the G_s subtype protein stimulates cAMP production, and the G_q subtype activates the phosphoinositol signaling pathway. Binding of ligand to receptors induces a conformational change in the transmembrane structure, leading to modifications of the intracellular structure of the receptor. This initiates the interaction with the G-protein in such a way that it dissociates from the activated receptor (Kieffer & Evans 2009). The G-protein, depending of its subtype, may further either directly affect potassium ion channels, like the invaring-rectifying potassium channel (Kir3) and G-protein-activated inwardly rectifying potassium channels (GIRKs), leading to a hyperpolarization of the neuron, or, act through several second messenger systems, having more long term effects on ion channels and/or genomic factors (Lambe & Aghajanian 2005; Kieffer & Evans 2009).

1.1.3 Pharmacological effects of opioids

The existence of different opioid receptors throughout the nervous system indicates that there are multiple neuronal sites and systems where endogenous or exogenous opioids can modulate their effects. Morphine acts principally as a μ -opioid receptor agonist, although it also has some activity through the δ -opioid receptor (1.9%) and κ -opioid receptors (0.6%) (Aderjan & Skopp 1997). CNS structures, such as striatum, nucleus accumbens (NAc), diagonal band of Broca, globus pallidus, ventral pallidum, bed nucleus of the stria terminalis, medial and cortical amygdale, mammillary nuclei, median raphe, locus coeruleus, nucleus of the solitary tract and most thalamic nuclei etc, are regions coupled to e.g. reward/reinforcement, intoxication, nociception/pain, stress and neurohormone secretion, and have an especially high abundance of μ -opioid receptors (Aderjan & Skopp 1997; Koob & Moan 2006;

McClung 2006). Upon binding of the ligand, μ -opioid receptors induce hyperpolarization and mediate inhibition of neural activity in several of these structures. In relation to the rewarding properties of opioids, activation of μ -opioid receptors leads to inhibition of neurones that release γ -aminobutyric acid (GABA) and tonically inhibit dopaminergic neurons in the ventral tegmental area (VTA). This leads to a surge of dopamine in the NAc and other mesolimbic-mesocortical brain regions, which is critical for the manifestation of reward (Johnson & North 1992; De Vries & Shippenberg 2002; Kreek *et al.* 2002; Luscher & Ungless 2006; Kauer & Malenka 2007).

In the nervous system, opioids modulate nociception through activation of peripheral and central μ , δ , and κ -receptors. The antinociceptive effect is mediated by at least two mechanisms: first, the excitability of the spinal primary neuron receiving the nociceptive input may be attenuated; second, the release of excitatory neurotransmitters, such as substance P, from afferent neurons may be inhibited by opioid receptor activation (Millan 1986; Stein 1993)

The morphine metabolite M6G is on the break of being introduced in the clinic as an analgesic. Its analgesic properties have been subjected for thorough investigation and found to be equivalent to morphine (Kilpatrick & Smith 2005). M6G has been shown to be 45 times as active as an analgesic compared to morphine sulphate when injected intracerebrally into mice, and a 37-fold increase with prolonged duration has been observed when given systemic (Shimomura *et al.* 1971; Boerner *et al.* 1975). M6G has been shown to have an improved side effect profile, with a reduced tendency to cause nausea, vomiting, sedation and respiratory depression compared with morphine (Kilpatrick & Smith 2005; Maurer *et al.* 2006). Several studies indicate that M6G penetrates the BBB less efficiently than morphine (Aasmundstad *et al.* 1995; Bickel *et al.* 1996; Aderjan & Skopp 1997; Wu *et al.* 1997). The transfer half life between plasma and effect compartment was determined to be 2.6 hours for morphine and 8.2 hours for M6G (Skarke *et al.* 2003; Kilpatrick & Smith 2005). Differences in permeability through cell membranes e.g. the BBB for morphine and M6G are believed to be associated with the lower lipid solubility of M6G, although differences in active influx and efflux by various transport proteins, such as the P-glycoprotein's, organic anion transporters, glucose transporters etc, may also be involved (Aderjan & Skopp 1997; Rook *et al.* 2006). The main differences between M6G and morphine that may be responsible for the different pharmacological and toxicological response are summarized by Kilpatrick *et al.* (2005) as follows:

- “Morphine has an slightly higher affinity for the μ -opioid receptor than M6G
- M6G shows slightly higher efficiency at the μ -opioid receptor
- M6G has a lower affinity for the κ -opioid receptor than morphine
- M6G has a very different absorption, distribution, metabolism, excretion (ADME) profile from morphine with:
 - A lower volume of distribution
 - A lower permeability across the BBB
 - Higher levels achieved in the extracellular compartment of the brain
 - A slow exit from the brain”

Kilpatrick *et al.* (2005) also emphasize in their review that there is significant amount of data that shows substantial differences between species related to M6G and morphine effects, and that extreme care should be taken when extrapolating results from non-primate studies to possible human effects.

The pharmacokinetic properties of M3G and M6G are quite similar. Volume of distribution (V_D) in mice are found to be 0.5, 0.9, and 3.2 l/kg, and clearance (CL) 24, 15, and 80 ml/min/kg for M3G, M6G, and morphine respectively (Handal *et al.* 2002). Effects of M3G administration in clinical studies have shown to include aversive effects like neuroexcitation (e.g. muscle twitches and seizures), hyperalgesia, and allodynia (Skarke *et al.* 2005; Rook *et al.* 2006). Measurement of locomotor activity after administration of a drug is a widely used method to measure psychostimulating properties. Both morphine and M6G has been shown to increase locomotion, whereas M3G shows no effect on this behaviour (Handal *et al.* 2002). Studies using the conditioned place preference (CPP) have shown that both morphine and M6G can have rewarding effects, whereas M3G shows a tendency to cause aversion (Vindenes *et al.* 2006). M3G has a very low affinity for opioid receptors compared to morphine and M6G (Bickel *et al.* 1996; Skarke *et al.* 2005), and its antagonistic effects compared to morphine and M6G is thus believed to be mediated through other mechanisms. This behavioural studies indicates that M6G as morphine may have rewarding properties, which would be mediated through increased dopamine release in NAc. Since M3G shows such opposite effects on behaviour, it is likely that M3G does not affect the dopamine functionality.

Opiates have a variety of secondary effects, like nausea, vomiting, sedation, depression of the respiratory system, as well as development of tolerance. Depression of the respiratory related neurons by agonist activity on μ -opioid receptors and δ -opioid receptors in ventrolateral medulla and dorsolateral pons is the main reason for suffocation by overdoses of heroin and morphine (Lalley 2008).

1.2 The nervous system underlying opioid addiction

1.2.1 The mesocorticolimbic dopamine system

Most dopamine containing neuron systems develop from an embryonic cell group originating at the mesencephalic-diencephalic region of the brain (midbrain dopaminergic neurones), and projects to various forebrain structures. One of these systems, the mesocorticolimbic system, consists of two diffuse, overlapping neuronal pathways, the mesolimbic and the mesocortical system, originating in the VTA. The mesolimbic system is defined by neurons projecting mostly to NAc and olfactory tubercle (ventral striatum), but also with projections to the septum, amygdala and hippocampus. On the NAc, these neurons project on GABAergic neurons. The mesocortical system originates in the medial VTA, with projections to the medial prefrontal, cingulate and perirhinal cortex (Kalivas & Volkow 2004; Kelley 2004; Everitt & Robbins 2005; Luscher & Ungless 2006; Pierce & Kumaresan 2006). Neurons in the mesolimbic system containing dopamine or dopamine receptors have been shown to be involved in rewarding responses to natural reinforcers, such as food, drink, social interaction and sex, as well as to several drugs (Wise & Rompre 1989; Nestler 2001a; Kreek *et al.* 2002; Kalivas & Volkow 2004; Wise 2004; Nestler 2005).

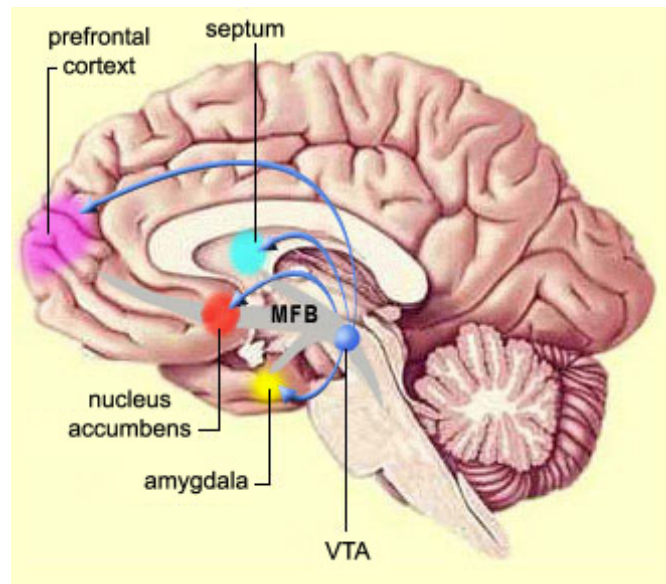


Figure 1. Blue arrows illustrate dopaminergic afferent projections originating in the VTA. The medial forebrain bundle (MFB) is the main circuit involved in reward, VTA and NAc lying along this circuit. Not shown in this figure are the glutamatergic and GABAergic interneurons also essential for this circuit.

GABAergic neurons on the NAc are activated by cortical inputs, mediated mainly by the excitatory neurotransmitter glutamate acting on N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxyl-5-methylisoxazole (AMPA) receptors. The degree of activity of Glutamate on these receptors is the principal source for long-term plasticity, a mechanism essential for learning. The coexistence of dopaminergic and glutaminergic innervations on the same dendrite spine of the GABAergic neurons is a critical factor for the effects on neural plasticity in NAc. Upon activation of both dopamine (D_1 subtype), AMPA and NMDA -receptors, a cascade of second messenger systems is activated, leading to up- and downregulation of numerous genes by different transcription factors like Δ FosB and CREB (cAMP response-element binding protein) working as coincidence detectors. Eventually, these processes will lead to synaptic remodelling together with changes in other effector mechanisms. (Konradi *et al.* 1996; Hyman & Malenka 2001; Nestler 2001b; Nestler 2001a; Kelley 2004; Nestler 2005).

Glutamate is released as respond to relative specific input from sensory, motor and memory circuits. Dopamine in NAc, on the other hand, is released upon unpredicted, rewarding events, e.g. food, sex, social interaction, drugs. Thus, the interaction of these two systems are essential for the shaping of neural networks associated with learning and memory of natural

rewards and/or various drugs, being the time frame of the interaction upon these receptors a crucial factor (Kelley 2004).

1.2.2 Dopamine and dopamine receptors

Dopamine is a catecholamine neurotransmitter, as well as an intermediate in the biosynthesis of other catecholamines like adrenaline and noradrenalin. The rate limiting step in dopamine synthesis is the conversion of L-tyrosine to L-DOPA (3, 4-dihydroxyphenylalanine) by tyrosine hydroxylase (TH), with tetrahydrobiopterin (BH₄) as cofactor (Venter *et al.* 1988; Kreek *et al.* 2002). TH is also subject for end-product inhibition by dopamine itself or other catecholamines by competing for a binding site with BH₄. TH has the property of existing in two affinity states, low and high, depending on neural activity and autoreceptor inhibitory feedback. This regulation ensures that the synthesis of dopamine is sufficient in activated neurons, as well as suppressed when dopamine is released in such amounts that it activates autoreceptors. L-DOPA is further converted to dopamine by DOPA decarboxylase, with pyridoxal phosphate as cofactor. The whole synthesis takes place in the cytoplasm, and the synthesised dopamine is actively transported into vesicles by the vesicular monoamine transporter (VMAT). This vesicular storage prevents both degradation by monoamine oxidase (MAO) and end-product inhibition of TH (Venter *et al.* 1988).

Upon neuronal activation, dopamine is released to the synaptic cleft by exocytosis and fusion with the synaptic terminal membrane. Released dopamine diffuses in the extracellular space, where its concentration is affected by reuptake, metabolism and binding to receptors. Dopaminergic receptors are metabotropic and pertain to the same family as the opioid receptors cited above (Kieffer & Evans 2009). There are five subtypes, D₁ to D₅, encompassed in two families. The D₁ and D₅ subtypes are members of the D₁-like family, and are coupled to G_s proteins, therefore stimulating adenylate cyclase (AC) and leading to an increase in cAMP production. The D₂, D₃, and D₄ are constituents of the D₂-like family, and coupled to G_i proteins, inhibiting AC and cAMP production. The main post synaptic dopamine receptors are of the D₁ or D₂ subtype, the main dopamine presynaptic autoreceptor being of the D₂ type (Kebabian & Calne 1979; Sokoloff *et al.* 1990; Sunahara *et al.* 1991; Van Tol *et al.* 1991; Lambe & Aghajanian 2005). Typical for metabotropic receptors, they work by modulatory mechanisms with a relative long delay between ligand-receptor interaction and functional changes in the recipient cell. The end result, with mechanisms and effects differing

between activation of D₁ and D₂, is modulation of membrane excitability. A tonic dopamine activation of D₁ generally increases membrane excitability, whereas D₂ receptor activation decreases the excitability of the neuron (Venter *et al.* 1988; Schultz 2002; West & Grace 2002; Di Chiara & Bassareo 2007).

1.2.3 Learning, reward and addiction

Associative learning of rewarding behaviors is mediated through a cognitive awareness in contingency between the action and the outcome which gives a rewarding sensation, which is mediated, at least in part, by increased dopamine levels in NAc (Schultz 2002; Kelley 2004). Drugs of abuse, also opiates, increase the extracellular levels of dopamine in the same way as natural factors such as food and sex, a process evolved for learning habits that increase the chance of survival of the subjects. Drugs act directly on these neural circuits involved in reward, and much more strongly than natural rewards, also bypassing the feedback mechanisms normally controlling dopamine release in NAc after the influence of natural rewards or associated cues. By greatly increasing dopamine levels, drugs promote Pavlovian incentive learning to drug conditioned stimuli through the neural plasticity mechanisms cited above, that is, dopamine and Glutamate activation of D₁, AMPA and NMDA receptors on neural dendrites in NAc (Kelley 2004; Di Chiara & Bassareo 2007). Repeated opiate use alters the activity of GABAergic neurons in NAc that are primary target by afferent dopamine and glutamate neurons. These GABAergic efferent neurons, which also contain the opioid peptides enkephalin or dynorphin, this last coexisting with substance P, project back upon the ventral pallidum and VTA, creating a feed back circuit (De Vries & Shippenberg 2002). Alterations in this system may contribute to the aversive consequences of opiate withdrawal (Volkow *et al.* 2008). Evidence for this is that treatment with a dopamine D₂ receptor agonist reduces opiate withdrawal effects, whereas an antagonist induces withdrawal signs (De Vries & Shippenberg 2002; Schultz 2002). A possible molecular mechanism is that activation of D₂ (Gi) receptors on GABAergic neurons in NAc reduce the excitation of these neurons, thus reducing GABAergic inhibition of the dopaminergic VTA neurons. An altered (reduced) tonic release of dopamine to NAc, as result of chronic drug abuse, will increase the activity of the inhibitory effects of GABAergic neurones and mediate withdrawal symptoms (De Vries & Shippenberg 2002).

1.3 The microdialysis technique

Brain dialysis was developed during the 1960's and 70's to measure extracellular levels of neurotransmitters among other substances *in vivo*. The dialysis technique has provided biochemical evidence of the involvement of dopamine in the rewarding effects of drugs of abuse like opiates, ethanol, nicotine, cocaine, amphetamines, cannabinoids, MDMA etc. (Di Chiara & Imperato 1988; Zocchi *et al.* 2003; Fadda *et al.* 2005).

Microdialysis is an *in vivo* method extensively used for measurement of endogenous neurotransmitters in various brain regions. A probe with a semi-permeable membrane is stereotactically implanted in the brain and perfused with a solution with similar ionic concentration as the cerebral spinal fluid. A concentration gradient is build between the extracellular space (ECS) and the inner of the probe, thus small molecules, like the neurotransmitters e.g. dopamine, will diffuse through the membrane to the inside of the probe following the concentration gradient. By regulating the flow through the probe, collection of the dialysis samples are possible and concentrations of neurotransmitters can be later measured with, e.g. by high performance liquid chromatography (HPLC) method connected to a detector (electrochemical, fluorescent, etc.) (Torregrossa & Kalivas 2008).

1.4 Aim of the study

The introduction in the clinic of M6G as an analgesic, as well as the importance of developing new treatments for addictive diseases, emphasize the need for further understanding of the underlying neurobiological mechanisms of opioids and their metabolites. The effects of the morphine metabolites M6G and M3G on analgesia (Kilpatrick & Smith 2005), locomotor activity (Handal *et al.* 2002), and reward (Abbott & Franklin 1991; Vindenes *et al.* 2006; Vindenes *et al.* 2008) have already been studied. However, the effect of M6G and M3G on mesocorticolimbic dopamine levels has, to our knowledge, not yet been studied.

Rodents metabolize morphine to M3G but not M6G (Wu *et al.* 1997; Handal *et al.* 2002), making them suitable for studying separately the effects seen after administration of morphine and M6G. The C57BL/6 mouse strain is selected for its known sensitivity for morphine, and for comparison to other studies done on the same strain at the Norwegian Institute of Public Health on effects of morphine, M6G and M3G e.g. (Handal *et al.* 2002; Fadda *et al.* 2005; Vindenes *et al.* 2006; Vindenes *et al.* 2008). Mice are also extensively used as model organism for the analysis of effects of a large number of drugs (Handal *et al.* 2002; Fadda *et*

al. 2005; Vindenes *et al.* 2006; Vindenes *et al.* 2008). E.g. did Murphy *et al.* (2001) perform a central study comparing locomotor activity and mesolimbic dopamine release in C57BL/6J mice after morphine administration. By using in vivo microdialysis, they showed a correlation between these two parameters. This study aims to further investigate the effects and participation of M6G and M3G on extracellular dopamine levels in NAc using the microdialysis method.

Morphine has well known rewarding properties. We wished to test our hypothesis that M6G, like morphine, mediate reward through elevated dopamine levels in NAc, an essential mediator of reward. Our second object is showing that M3G has no effect on dopamine levels, providing evidence and partly explaining the lack of behavioural changes in locomotion and CPP studies.

2. Materials and methods

2.1 Animals and conditions

Male C57BL/6 mice (n=49) (Bomholt, Denmark) weighing about 20-30 g with age between 6-8 weeks, were used. They were housed six mice per cage, with free access to food and water. The cages were placed in standard housing environmental conditions, with artificial 12 hour light:dark cycle, light period from 08:00 to 20:00. Before the experiment, mice were left for about one week for acclimatization to their new housing conditions.

After implantation of the guide cannula (see below), the mice were placed individually in a special cage in order to avoid damage to the mouse or the implant. Personal experience from other scientists revealed that implanted mouse would attack and damage the implant and each other if housed together.

The experiments in this study were performed in accordance with the Norwegian regulations on animal experimentation (the Norwegian “Animal Welfare Act” and the “Regulation on Animal Experimentation”) and after approval by the Norwegian National Animal Research Authority.

2.2 Surgical procedure

Mice were weighted and anesthetized with a 10 ml/kg mixture containing 10 mg/ml Ketalar and 1 mg/ml Xylazine (corresponding to a dose of 100 and 10 mg/kg respectively) administered intraperitoneally (i.p.). The level of sedation during surgery was checked with a pinch on the tail or foot and supplemental doses of anesthetic were administered when required. Once completely anesthetized, the animal was placed in a stereotaxic apparatus (Model 900 with 926 mouse adaptor, David Kopf Instruments, Tujunga, CA, USA), shown in figure 3. During the surgical procedure, the body temperature was kept constant at 37.5 °C by a rectal probe connected to a temperature controller (CMA/150, CMA Microdialysis, Solna, Sweden) regulating a thermal pad situated under the animal's body. The eyes were covered with eye salve to reduce the risk of keratitis or other damages to the eyes.

Once the animal was in place, the skull was exposed by making a longitudinal incision through the skin at the upper side of the head, and the skull bone was cleaned with alcohol. The elevation of the nose clamp fixing the head was adjusted until lambda (the intersection between the lambdoid and the coronal sutures) and bregma (the intersection between the sagittal and the coronal sutures) were at the same horizontal plane. A hole in the skull was drilled in a dorsal position over the NAc at the following coordinates relative to bregma: Anterior (A): + 1.4 mm; Lateral (L): \pm 0.8 mm (Paxinos & Franklin 2001). The lateral coordinates (right or left) were switched every other operated animal. Skull structures are shown for clarification in figure 2. Another cavity was made at a posterior position at the contralateral side of the skull relative to the sagittal suture. This cavity was used to fix a screw which will help to stabilize the implant.

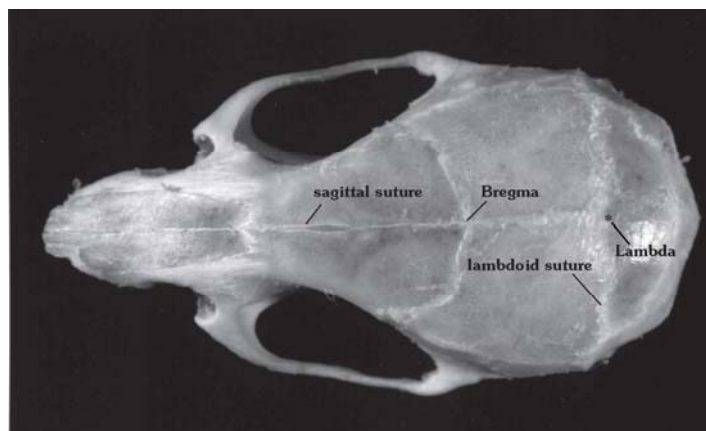


Figure 2. Picture showing the dorsal surface of the mouse skull, with indication of the structures used as reference points in this study (Paxinos & Franklin 2001).

A holder bar carrying a vertical guide cannula (CMA/7 Guide Cannula, CMA Microdialysis, Solna, Sweden) was fixed to the stereotactic manipulator. The guide cannula was lowered through the hole into the brain to a coordinate 4.0 mm ventral (V) relative to bregma. Dental cement (Dentalon Plus, Heraeus Kulzer GmbH, Hanau, Germany) was used to fix the guide cannula to the skull. The dental cement encompassed also the fixation screw and a bolt for connection to a wire (Figure 3). After surgery, the mice were placed in separate cages for at least 24 hours for recovery.



Figure 3. Mice placed in the stereotaxic apparatus. The head is fixated by ear bars and a nose clamp. The dental cement around the implant can be seen over the head.

2.3 In vivo microdialysis experiment

At least 24 h after implantation, each mouse was attached to the microdialysis sampling equipment. To ease the connection procedure, the mice were slightly anesthetized using approximately 0.08 ml of the anesthetic mixture. After removing the stylet (dummy probe) sealing the guide cannula, a CMA 7/1 microdialysis probe (CMA Microdialysis, Solna, Sweden), consisting of a 7 mm shaft with a diameter of 240 μm and a 1 mm long semipermeable membrane, was inserted through the guide cannula. Once in place, only the 1mm semi permeable membrane was protruding beyond the guide cannula and entering the area of interest (NAc). One end of a 30 cm steel wire was attached to the metallic bolt encased in the implant. Both the probe inlet and outlet were connected to the channel ports of a dual channel swivel (Model 375/D/22QE, Instech Solomon, Plymouth Meeting PA, USA) attached to a multi-axis counter-balanced lever arm (Model SMCLA, Instech Solomon, Plymouth Meeting PA, USA) situated at the top of the experimental cage. The other end of the steel wire was fastened to a clamp in the swivel, transmitting the movement of the animal to the swivel and arm mounting, impeding the twisting of the tubes (figure 4). The inlet channel was connected to a syringe infusion pump (CMA 400, CMA Microdialysis, Solna, Sweden) containing an artificial cerebrospinal fluid (Ringer's solution: 148 mM NaCl, 2.7 mM KCl, 1.2 mM CaCl_2 , 0.85 mM MgCl_2). The outlet channel was likewise connected to a refrigerated fraction collector (CMA/170, CMA Microdialysis, Solna, Sweden), where samples were collected in glass vials without any preservatives and maintained at 6 °C during the experiment. The pump flow was set to 0.2 $\mu\text{l}/\text{min}$, and mice left for conditioning over night.



Figure 4. Picture showing a mouse on the microdialysis experimental cage with the microdialysis probe attached to the pump (on the background) and the fraction collector (partially seen at the side). The multi-axis counter-balanced lever arm with the swivel can be seen at the top of the cage.

2.4 Experimental protocol

On the following day, the pump flow was increased to 1 $\mu\text{l}/\text{min}$. The fraction collector, chilled to 6 °C, was programmed to let the first 45 min of flow-through go to waste, ensuring that the first sampling would not contain solution that had been standing in the probe-swivel loop over night. Thereafter four baseline samples were collected during 20 minutes each. Immediately after, the mice received their respective treatment and sampling continued with the collection of nine 20 minutes samples.

Treatments consisted of either morphine 10 or 30 $\mu\text{mol}/\text{kg}$ (n=10 and 9, respectively), M6G 10 or 30 $\mu\text{mol}/\text{kg}$ (n=8 and 6, respectively), M3G 100 $\mu\text{mol}/\text{kg}$ (n=9), or physiological saline solution (n=7). All treatments were given subcutaneously (s.c.) on the back about 1 cm cranial from the tail root.

2.5 Chromatographic analysis of brain samples

After the last sample was collected, the vials with the samples were sealed and transported in a chilled container and placed in the HPLC for analysis. 18 μl of each sample were injected either on a Dionex Ultimate 3000 HPLC system (Dionex Corporation, Sunnyvale, CA) or on a system consisting of an Agilent model 1100 series pump (Agilent, Santa Clara, CA, USA) and a CMA/200 autosampler (CMA Microdialysis, Solna, Sweden). In both cases, the mobile

phase was composed of 150 mM sodium dihydrogen phosphate, 4.76 mM citric acid, 3 mM sodium dodecyl sulphate, and 50 μ M EDTA dissolved in 10 % methanol and 15 % acetonitrile, adjusted at pH 5.6, and running at a flow-rate of 0.7 ml/min. Separation was achieved through a 80 x 3.2 mm ESA silica column HR, filled with 3 μ m particles with pore size 120 Å and operated at either 30 °C or room temperature (about 22 °C). In each HPLC system, detection and quantification of DA was accomplished with a Coulochem II electrochemical detector (ESA Inc., Chelmsford, MA) using an ESA model 5020 guard cell (situated between the pump and the auto-injector) operating at 500 mV, and an ESA model 5014B analytical cell operating at -100 mV (E1) and 300 mV (E2). Standard calibration samples were injected both at the beginning and the end of each analytical sequence. Chromatographic data were analysed and integrated with a Dionex Chromeleon chromatographic software (version 6.80), using the height of the peaks for quantification.

2.6 Histological verification of probe localization

At the end of the experiment, mice were deeply anesthetized with an i.p. injection of the ketamin/xylazine mixture, and detached from the microdialysis equipment, including removal of the microdialysis probe. Each mouse was then immediately sacrificed, the brain gently separated from the skull and frozen either in a mixture of dry ice and isopentane (temperature ca. -78 °C) or on liquid nitrogen, and stored at -20 °C.

Brain slices, 12 -14 μ m thick, were later obtained using a cryostat HM 550 (Microm International GmbH, Walldorf, Germany). The slices were stained with methylene blue. Probe location in each brain slice was checked by microscopic analysis and probe positions compared in relation to a well known mouse brain atlas by Franklin and Paxinos (2001) (figure 5).

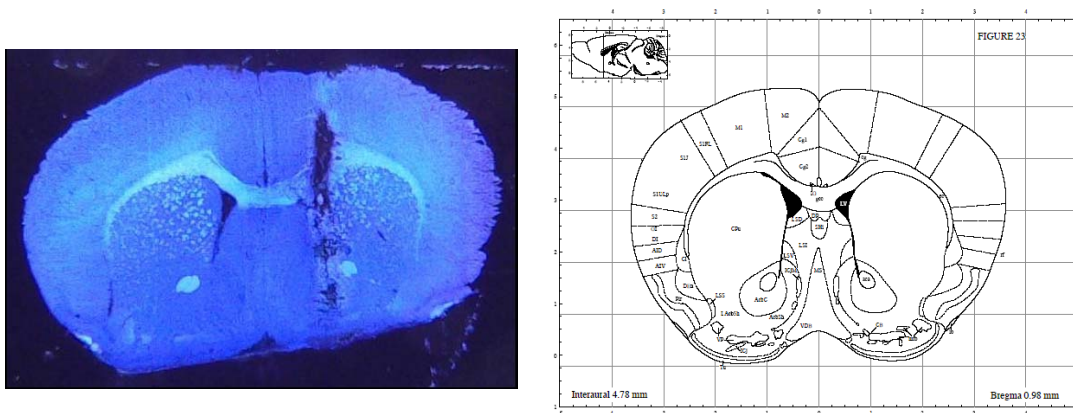


Figure 5. Methylene blue stained brain slice and the corresponding plate of the atlas (Paxinos & Franklin 2001). The track of the implanted probe can be distinguished on the right hemisphere, with its lower 1 mm, encompassing the membrane, inside the limits of the NAc.

In order to overcome some problems in locating the correct probe position, halfway into the experimental period we started to color the probe location. After disconnection from the microdialysis equipment and before sacrificing, a microdialysis probe, detached of its dialysis membrane, was inserted through the guide cannula. Diluted methylene blue, 2 μ l, was injected directly into the brain, coloring the location where the probe had been situated during the experiment. Thereafter, the procedure followed as explained above.

2.7 Drugs

Morphine hydrochloride was purchased from Norsk Medisinaldepot, Oslo, Norway. Morphine-6- β -D-glucuronide hydrate and morphine-3- β -D-glucuronide hydrate were purchased from Lipomed, Arlesheim, Switzerland. All drugs were diluted in physiological saline to a final concentration resulting in an injection volume of 0.01 ml/g mouse weight.

The doses of 10 and 30 μ mol/kg morphine and M6G were chosen since previous studies have shown effects of such doses in behaviour models on locomotion and/or CPP. M3G dose was set at 100 μ mol/kg, after experiencing the death and feebleness of several mice after injection of the initial doses of 200 and 400 μ mol/kg, doses previously used in behaviour models (Vindenes *et al.* 2006).

2.8 Data analysis

In order to compensate for differences in basal levels of dopamine between individual mice, dopamine was transformed to the percentage of the average of the last three baseline samples. The results were analyzed with Linear Mixed Models, using SPSS 15.0, with drug treatment as dependent factor and time as covariate. The mixed model is fitted for repeated data, with compound symmetry as covariate matrix model (selected by the lowest Akaike's information criterion). The significances from "Type III Tests of Fixed Effects" were used to examine the overall effect of drug treatment and time, as well as their interaction. The significances from the "Estimates of Fixed Effects" were used as test for differences between each treatment dose and saline. A significance p-value lower than 0.05 was considered as statistical significant.

3. Results

3.1 Dopamine concentrations

Measurement of the basal levels of dopamine in NAc gave a mean value of 0.72 ± 0.12 pg/ μ l. Saline treatment shows no effect on the extracellular dopamine concentration in NAc compared to baseline values (figures 6, 7 and 8).

For the morphine treatment, the mixed model shows a significant interaction between drug treatment and time ($p < 0.001$). It also reveals a significant increase of extracellular dopamine after morphine 10 and 30 μ mol/kg treatments compared to the saline treatment ($p < 0.001$ and $p < 0.017$ respectively) (figure 6).

Treatment with M6G 10 and 30 μ mol/kg gives the same significant interaction between drug treatment and time ($p < 0.001$). Both doses showed a significant increase in extracellular dopamine in NAc compared to the saline treatment ($p < 0.001$ and $p < 0.004$ respectively) (figure 7).

No significant different main effect on dopamine levels was seen after the treatment with M3G 100 μ mol/kg ($p < 0.316$). However, there was a significant interaction between treatment and time ($p < 0.008$), as well as a significant difference between M3G and saline treatment ($p < 0.011$) (figure 8). However, additional 2-tailed t-tests for independent samples for each time point did not show any significant difference between M3G and saline treatment.

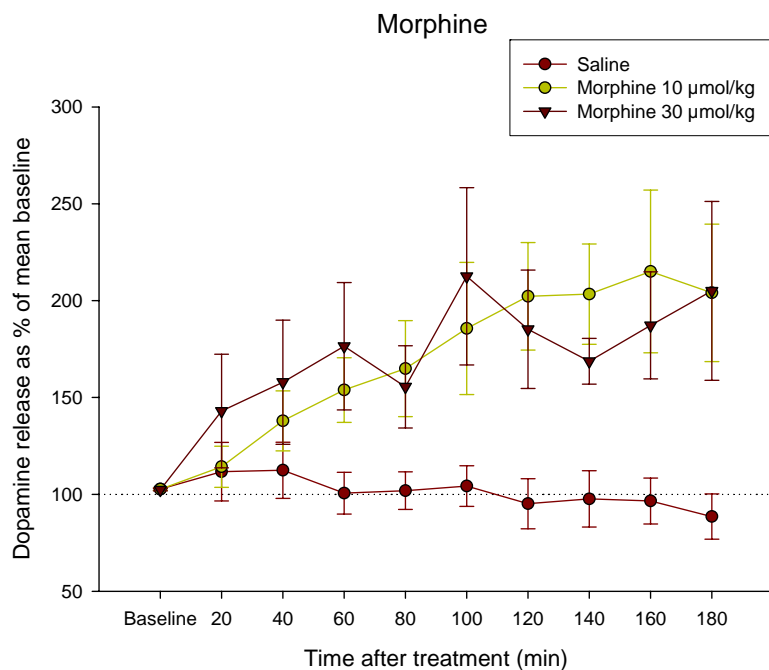


Figure 6. Changes in extracellular concentrations of dopamine (expressed as percentage of baseline) after the s.c. administration of saline (brown circles), or 10 µmol/kg (green circles) or 30 µmol/kg (dark brown triangles) morphine. Values are mean \pm s.e.m. Baseline represents the average of the three samples taken before drug administration.

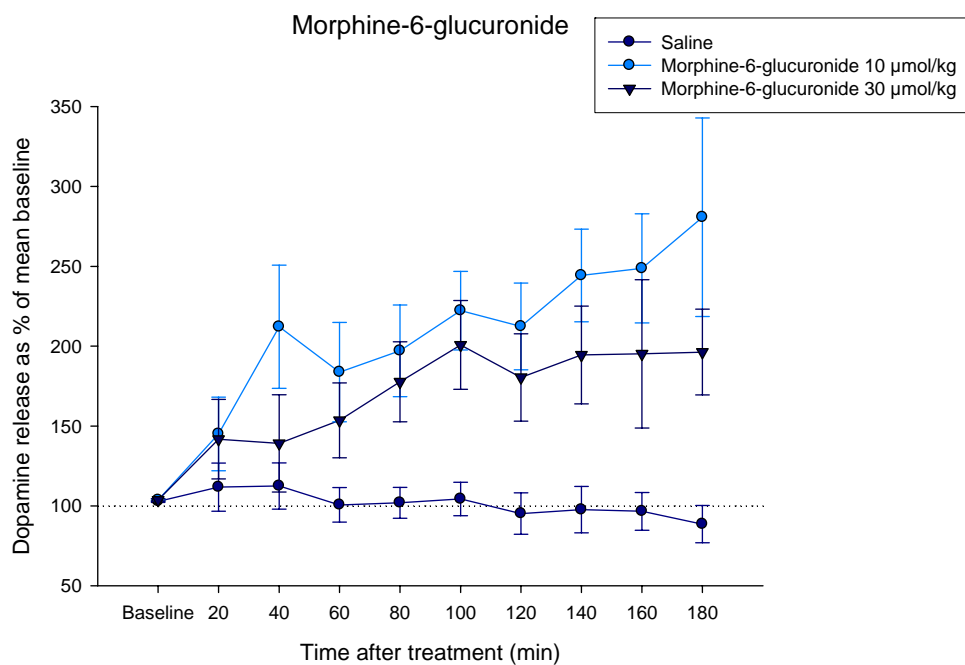


Figure 7. Changes in extracellular concentrations of dopamine (expressed as percentage of baseline) after the s.c. administration of saline (dark blue circles), or 10 µmol/kg (blue circles) or 30 µmol/kg (indigo triangles) M6G. Values are mean \pm s.e.m. Baseline represents the average of the three samples taken before drug administration.

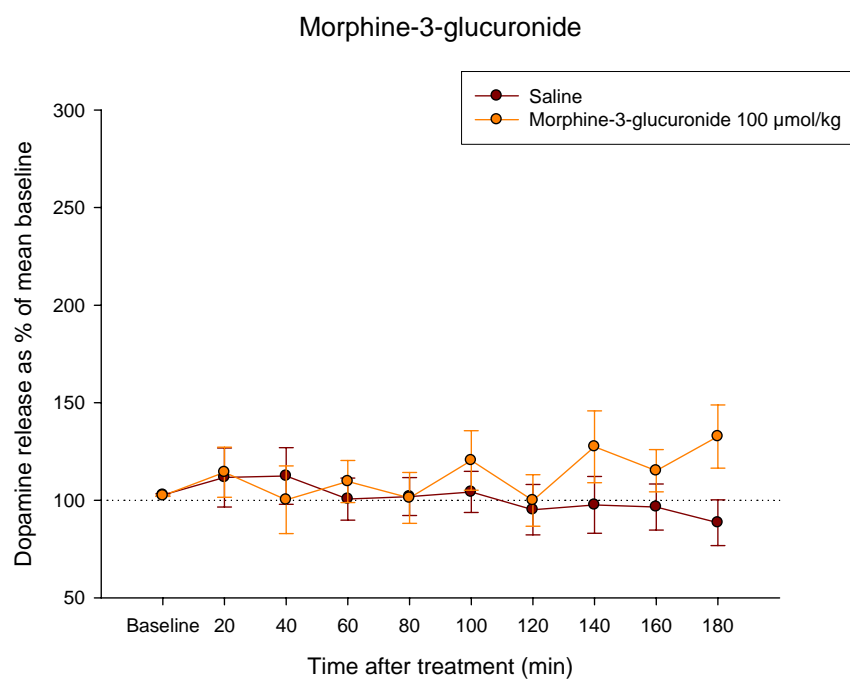


Figure 8. Changes in extracellular concentrations of dopamine (expressed as percentage of baseline) after the s.c. administration of saline (brown circles), or 100 µmol/kg (orange circles) M3G. Values are mean \pm s.e.m. Baseline represents the average of the three samples taken before drug administration.

3.2 Histological verification of probe localization

The location of the microdialysis probes was verified in all animals (n=49), and is illustrated in figure 9. All probes were located within the NAc, between the antero-posterior coordinates + 0.74 mm and + 1.42 mm relative to bregma (Paxinos & Franklin 2001).

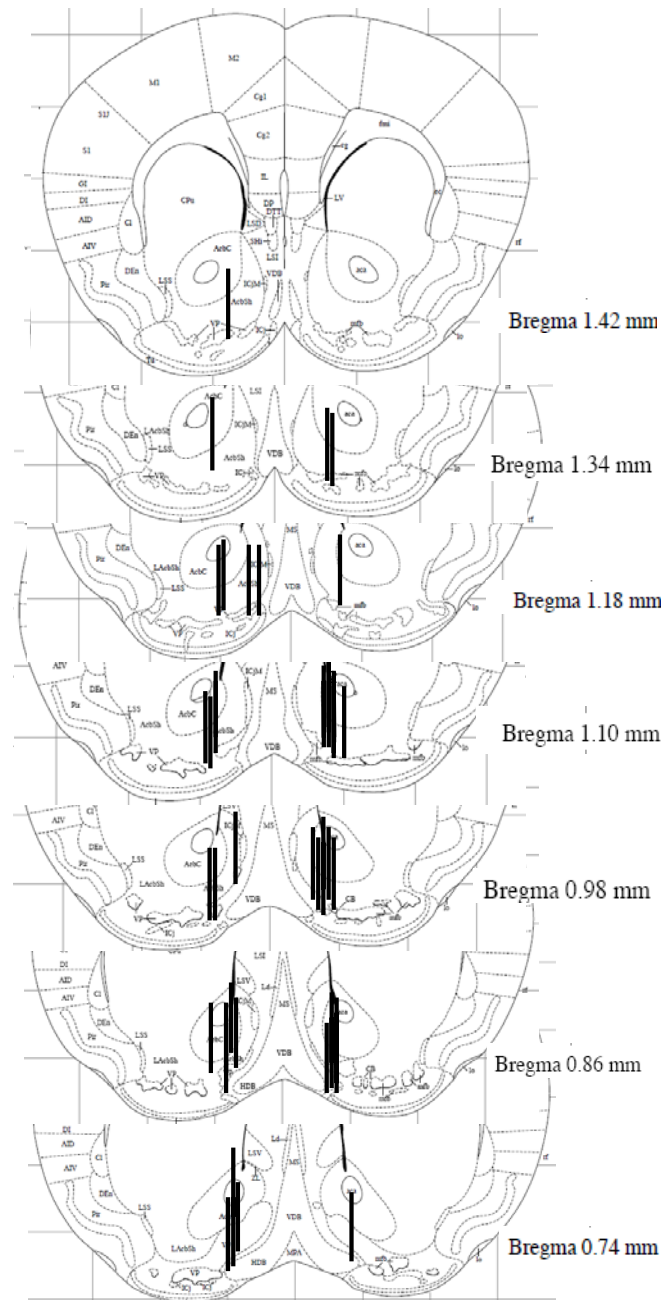


Figure 9. Schematic representation of the microdialysis probes with reference to the atlas of Paxinos & Franklin. Vertical lines represent the length of the membrane (1 mm).

4. Discussion

4.1 Purpose

Morphine is known to increase dopamine levels in the NAc, an effect related to its reinforcing effects (Zocchi *et al.* 2003; Fadda *et al.* 2005; Kalivas & O'Brien 2008). The purpose of this study was to determine if M3G and M6G also have similar effects on extracellular dopamine in NAc. This study is a supplement to other studies done at our laboratory using behavioral models, like the CPP model (Handal *et al.* 2002; Vindenes *et al.* 2006; Vindenes *et al.* 2008) and locomotor activity (Handal *et al.* 2002; Vindenes *et al.* 2006; Vindenes *et al.* 2008), as a measurement of the reinforcing and stimulating effects of M3G and M6G, behaviors known to be associated to extracellular dopamine levels in NAc.

4.2 Choice of method

Microdialysis is a method which allows researchers to monitor neurochemical changes after drug treatment or other interventions. This technique has been widely used to increase the knowledge about the neurobiological processes underlying addiction. Torregrossa *et al.* (2008) have published an excellent review emphasizing the importance of the microdialysis technique in determination of the ultimate alterations in various neurotransmitter systems, e.g. glutamate, GABA, dopamine, and others, involved in addiction.

4.3 Opiate metabolites and dopamine release

The results obtained in this study reveal that M6G, like morphine, increases the extracellular levels of dopamine in NAc, whereas M3G treatment does not lead to such an increase. This is in accordance with previous observations of a stimulating effect of morphine and M6G, but not M3G, on locomotion (Handal *et al.* 2002; Vindenes *et al.* Submitted-b), as well as a reinforcing effect of morphine and M6G, and a tendency to aversion for M3G in CPP (Handal *et al.* 2002; Vindenes *et al.* 2006; Vindenes *et al.* 2008).

The increased dopamine observed after morphine treatment is also consistent with microdialysis studies done by Fadda *et al.* (2005) on effects of morphine on extracellular dopamine levels in rats, and similar studies by Zocchi *et al.* (2003) and Murphy *et al.* (2001) on mice. Fadda *et al.* (2005) observed also increased serotonin (5-HT) levels in NAc, and proposed the possible involvement of the serotonergic systems in the behavioral and biochemical responses to morphine. Thus, involvement of other neurotransmitter systems can also be important for the neurobiological effects of morphine and, probably, its metabolites.

The statistical analysis showed a significant interaction between M3G and time. However, the simple visual inspection of the curves (figure 8) shows that the curve for M3G is very close to the one for saline, and the significance could be due to the slightly higher values for M3G towards the end of the sampling period. That this significant effect could be due to chance can be supported by the fact that any of the t-test between M3G and saline values for each time, did not reach statistical significance. In an extension of this study, Vindenes *et al.* further completed the investigation with an additional dose of M3G 50 $\mu\text{mol/kg}$, as well as increasing some of the test groups with additional mice. Results are not yet published, but they show a decrease in dopamine for the M3G 50 $\mu\text{mol/kg}$ compared to saline. When this dose is included in the statistics, the main effect is still not significant for M3G, time and treatment interaction continues to be significant, but a no significant effect on dopamine compared to saline is observed (Vindenes *et al.* Submitted-a).

In behavioral studies, a delayed onset of effects has been detected for M6G. Grung *et al.* (1998) observed that the onset of increased locomotion is seen about 5 min later for M6G than for morphine, despite both reached the same level after 20 min. And Vindenes *et al.* (2008) experienced that a 15 min delay had to be introduced before conditioning in order to obtain a significant CPP for M6G, whereas morphine significantly induced CPP only when conditioning was performed immediately after drug injection. This is also in accordance with the observed delay of the antinociceptive effect for M6G compared to morphine, as reported in pain studies (Bouw *et al.* 2001; Skarke *et al.* 2003). The present study did not however show a delayed dopamine release for M6G compared to morphine, as would be expected from the behavioral studies cited above. This discrepancy can be explained by limitations in this microdialysis model. As a consequence of the extremely low quantity of dopamine in the extracellular fluid, the sample period had to be set to 20 min for acquiring a satisfying sample amount for analysis, a time period longer than the delay observed in the behavioral effects. A

shorter sampling interval possibly would have captured important information about the onset of dopamine release, especially in the first 20 min. The levels of dopamine after both morphine and M6G treatment also increase continuously throughout the 180 min measured. This is long after the maximal increase in the locomotor activity observed in mice (Handal *et al.* 2002). It may have been convenient to extend the experimental time to capture a possible drop in dopamine levels for comparison with the decreased locomotion and CPP over time. The results for M3G in this study are also consistent with behavioral studies. No effects of M3G have been seen either in locomotion studies or CPP studies, but a tendency to aversion was seen in the CPP model (Handal *et al.* 2002; Vindenes *et al.* 2006).

4.4 Methodological challenges

Several mice died, or noticeable feebleness was seen, after injections of M3G 200 and 400 $\mu\text{mol/kg}$, which were the initial doses chosen for M3G. No such effects were seen in previous behavioral studies using up to 500 $\mu\text{mol/kg}$ M3G (Handal *et al.* 2002; Vindenes *et al.* 2006). We assume that the surgical procedure and implantation of the microdialysis probe in some way affects the fitness of the animal and reduces the threshold for toxic effects. M3G has been shown, clinically and experimentally, to have neuroexcitatory side effects resulting in allodynia, myoclonus and seizures (Smith 1998).

At the beginning of the experiment, we experienced a disagreement between our results and similar studies on the effect of morphine on the dopamine response. After a thorough revision of the procedure, we concluded that a hypotonic perfusion solution, with a 10 times lower ionic concentration, had been used instead the customary physiological, isotonic solution. Robinson and Justice (Robinson & Justice 1991) emphasize the importance of using a perfusion solution which is as close as possible to that of the extracellular fluid with regard to the most important electrolytes. A hypotonic solution would much likely create a concentration gradient between the probe and extracellular fluid (ECF), thus draining the ECF of ions. This will cause an ionic imbalance at the neuronal membranes, affecting the neuronal activity and neurotransmitter release. This process would likely take place immediately after initiating the perfusion, that is, already the night before sampling. Figures 10 to 13 show the difference in the results between the hypotonic solution and the physiological correct solution used there after.

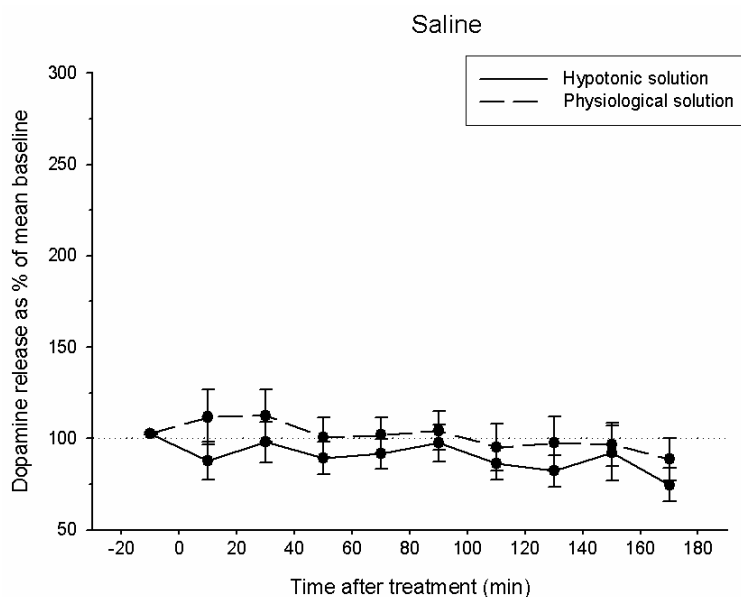


Figure 10. Changes in extracellular concentrations of dopamine (expressed as percentage of baseline) after the s.c. administration of saline in animals with the microdialysis probe perfused with either hypotonic buffer (continuous line) or physiological isotonic solution (dashed line). Values are mean \pm s.e.m. Baseline represents the average of the three samples taken before drug administration.

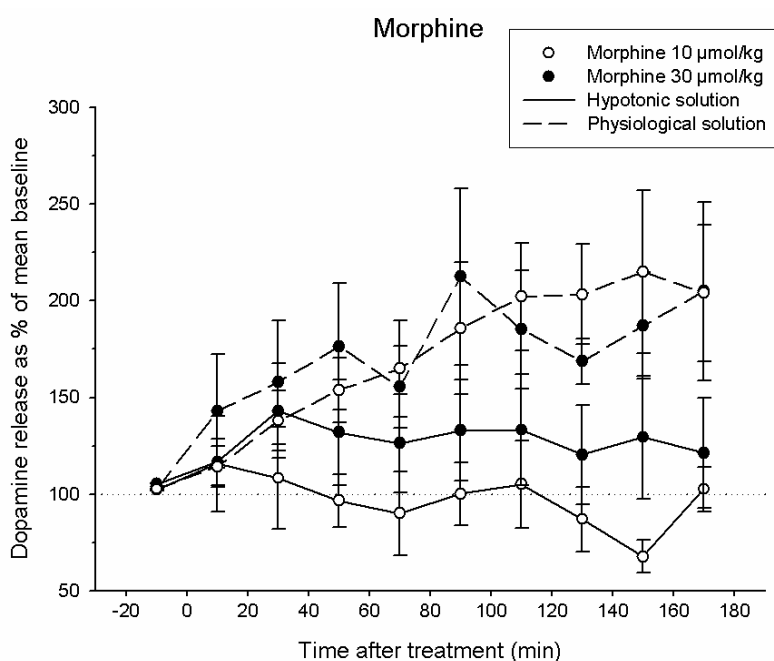


Figure 11. Changes in extracellular concentrations of dopamine (expressed as percentage of baseline) after the s.c. administration of morphine 10 μ mol/kg (open circles) or 30 μ mol/kg (closed circles) in animals with the microdialysis probe perfused with either hypotonic buffer (continuous line) or physiological isotonic solution (dashed line). Values are mean \pm s.e.m. Baseline represents the average of the three samples taken before drug administration.

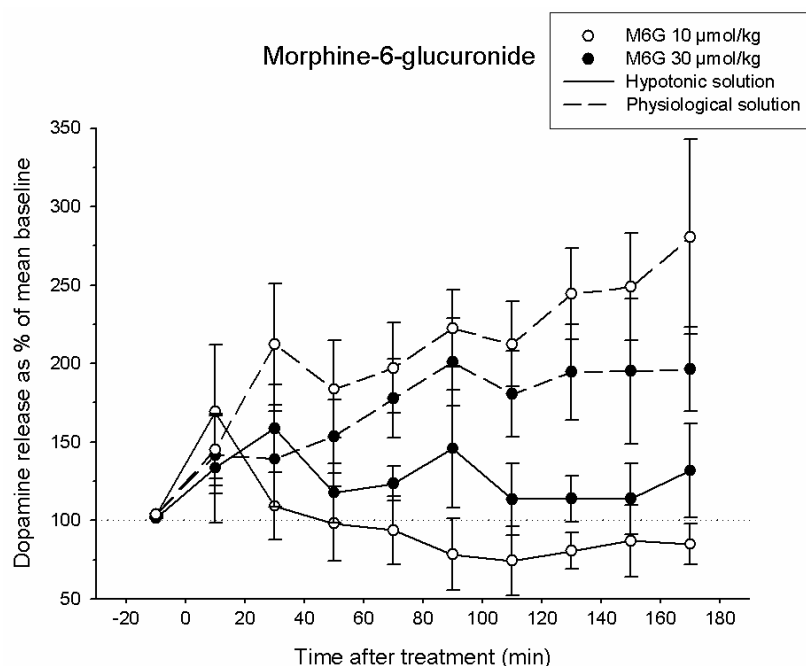


Figure 12. Changes in extracellular concentrations of dopamine (expressed as percentage of baseline) after the s.c. administration of M6G 10 µmol/kg (open circles) or 30 µmol/kg (closed circles) in animals with the microdialysis probe perfused with either hypotonic buffer (continuous line) or physiological isotonic solution (dashed line). Values are mean \pm s.e.m. Baseline represents the average of the three samples taken before drug administration.

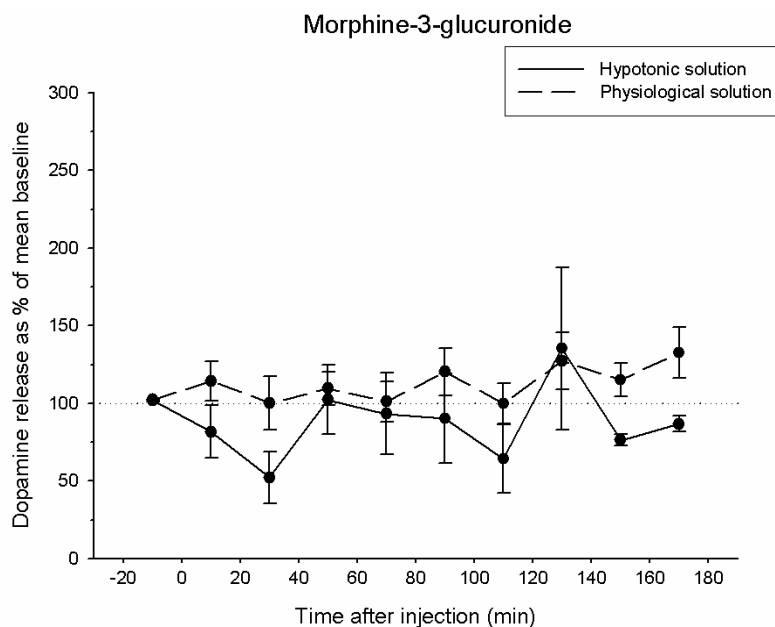


Figure 13. Changes in extracellular concentrations of dopamine (expressed as percentage of baseline) after the s.c. administration of M3G 100 µmol/kg in animals with the microdialysis probe perfused with either hypotonic buffer (continuous line) or physiological isotonic solution (dashed line). Values are mean \pm s.e.m. Baseline represents the average of the three samples taken before drug administration.

5. Conclusion

The results obtained in this study show that, in concordance with our hypothesis, M6G, like morphine, increases the extracellular levels of dopamine in NAc, while M3G treatment does not lead to such an increase. Since dopamine release on this area has been closely related to the reinforcing and rewarding effects of drugs of abuse, this study confirms previous results revealing rewarding properties of M6G similar to morphine. Therefore, M6G may have addiction potential and may contribute to the development of addiction and dependence of heroin and morphine.

6. References

- Aasmundstad TA, Morland J, Paulsen RE (1995) Distribution of morphine 6-glucuronide and morphine across the blood-brain-barrier in awake, freely moving rats investigated by in-vivo microdialysis sampling. *Journal of Pharmacology and Experimental Therapeutics* 275: 435-441
- Abbott FV, Franklin KBJ (1991) Morphine-6-glucuronide contributes to rewarding effects of opiates. *Life Sciences* 48: 1157-1163
- Aderjan RE, Skopp G (1997) Formation and clearance of active and inactive metabolites of opiates in humans. *5th International Congress of Therapeutic Drug Monitoring and Clinical Toxicology Conference Vancouver, Canada*. Lippincott Williams & Wilkins. 561-569
- Bickel U, Schumacher OP, Kang YS, Voigt K (1996) Poor permeability of morphine 3-glucuronide and morphine 6-glucuronide through the blood-brain barrier in the rat. *Journal of Pharmacology and Experimental Therapeutics* 278: 107-113
- Boerner U, Abbott S, Roe RL (1975) Metabolism of morphine and heroin in man. *Drug Metabolism Reviews* 4: 39-73
- Bouw MR, Xie RJ, Tunblad K, Hammarlund-Udenaes M (2001) Blood-brain barrier transport and brain distribution of morphine-6-glucuronide in relation to the antinociceptive effect in rats-pharmacokinetic/pharmacodynamic modelling. *British Journal of Pharmacology* 134: 1796-1804
- Coffman BL, Rios GR, King CD, Tephly TR (1997) Human UGT2B7 catalyzes morphine glucuronidation. *Drug Metabolism and Disposition* 25: 1-4
- De Vries TJ, Shippenberg TS (2002) Neural systems underlying opiate addiction. *Journal of Neuroscience* 22: 3321-3325
- Di Chiara G, Bassareo V (2007) Reward system and addiction: what dopamine does and doesn't do. *Current Opinion in Pharmacology* 7: 69-76
- Di Chiara G, Imperato A (1988) Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proceedings of the National Academy of Sciences of the United States of America* 85: 5274-5278
- Everitt BJ, Robbins TW (2005) Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. *Nature Neuroscience* 8: 1481-1489
- Fadda P, Scherma M, Fresu A, Collu M, Fratta W (2005) Dopamine and serotonin release in dorsal striatum and nucleus accumbens is differentially modulated by morphine in DBA/2J and C57BL/6J mice. *Synapse* 56: 29-38
- Grung M, Skurtveit S, Aasmundstad TA, Handal M, Alkana RL, Morland J (1998) Morphine-6-glucuronide-induced locomotor stimulation in mice: Role of opioid receptors. *Pharmacology & Toxicology* 82: 3-10
- Handal M, Grung M, Skurtveit S, Ripel A, Morland J (2002) Pharmacokinetic differences of morphine and morphine-glucuronides are reflected in locomotor activity. *Pharmacology Biochemistry and Behavior* 73: 883-892
- Hyman SE, Malenka RC (2001) Addiction and the brain: The neurobiology of compulsion and its persistence. *Nature Reviews Neuroscience* 2: 695-703

- Inturrisi CE, Max MB, Foley KM, Schultz M, Shin SU, Houde RW (1984) The pharmacokinetics of heroin in patients with chronic pain. *New England Journal of Medicine* 310: 1213-1217
- Inturrisi CE, Schultz M, Shin S, Umans JG, Angel L, Simon EJ (1983) Evidence from opiate binding studies that heroin acts through its metabolites. *Life Sciences* 33 Suppl 1: 773-776
- Johnson SW, North RA (1992) Opioids excite dopamine neurons by hyperpolarization of local interneurons. *Journal of Neuroscience* 12: 483-488
- Kalivas PW, O'Brien C (2008) Drug addiction as a pathology of staged neuroplasticity. *Neuropsychopharmacology* 33: 166-180
- Kalivas PW, Volkow ND (2004) The neural basis of addiction: A pathology of motivation and choice. *157th Annual Meeting of the American-Psychiatric-Association Conference New York*. Amer Psychiatric Publishing, Inc. 1403-1413
- Kauer JA, Malenka RC (2007) Synaptic plasticity and addiction. *Nature Reviews Neuroscience* 8: 844-858
- Kebabian JW, Calne DB (1979) Multiple receptors for dopamine. *Nature* 277: 93-96
- Kelley AE (2004) Memory and addiction: Shared neural circuitry and molecular mechanisms. *Neuron* 44: 161-179
- Kieffer BL, Evans CJ (2009) Opioid receptors: From binding sites to visible molecules in vivo. *Neuropharmacology* 56: 205-212
- Kilpatrick GJ, Smith TW (2005) Morphine-6-glucuronide: Actions and mechanisms. *Medicinal Research Reviews* 25: 521-544
- Konradi C, Leveque JC, Hyman SE (1996) Amphetamine and dopamine-induced immediate early gene expression in striatal neurons depends on postsynaptic NMDA receptors and calcium. *Journal of Neuroscience* 16: 4231-4239
- Koob GF, Moan LM (2006) **Neurobiology of addiction**. Elsevier, London, pp 121-159
- Kreek MJ, LaForge KS, Butelman E (2002) Pharmacotherapy of addictions. *Nature Reviews Drug Discovery* 1: 710-726
- Lalley PM (2008) Opioidergic and dopaminergic modulation of respiration. *Respiratory Physiology & Neurobiology* 164: 160-167
- Lambe EK, Aghajanian GK (2005) Using basic electrophysiology to understand the neurobiology of mental illness. In: Charney DS, Nestler EJ (eds) **Neurobiology of mental illness** Oxford University Press, New York
- Lotsch J (2005) Opioid metabolites. *Journal of Pain and Symptom Management* 29: pp 10-24
- Luscher C, Ungless MA (2006) The mechanistic classification of addictive drugs. *Plos Medicine* 3: 2005-2010
- Mark TL, Woody GE, Juday T, Kleber HD (2001) The economic costs of heroin addiction in the United States. *Drug and Alcohol Dependence* 61: 195-206
- Maurer HH, Sauer C, Theobald DS (2006) Toxicokinetics of drugs of abuse: Current knowledge of the isoenzymes involved in the human metabolism of tetrahydrocannabinol, cocaine, heroin, morphine, and codeine. *Therapeutic Drug Monitoring* 28: 447-453
- McClung CA (2006) The molecular mechanisms of morphine addiction. *Reviews in the Neurosciences* 17: 393-402

- Millan MJ (1986) Multiple opioid systems and pain. *Pain* 27: 303-347
- Murphy NP, Lam HA, Maidment NT (2001) A comparison of morphine-induced locomotor activity and mesolimbic dopamine release in C57BL6, 129Sv and DBA2 mice. *Journal of Neurochemistry* 79: 626-635
- Nestler EJ (2001a) Molecular basis of long-term plasticity underlying addiction. *Nature Reviews Neuroscience* 2: 119-128
- Nestler EJ (2001b) Molecular neurobiology of addiction. *American Journal on Addictions* 10: 201-217
- Nestler EJ (2005) Is there a common molecular pathway for addiction? *Nature Neuroscience* 8: 1445-1449
- Oldendor W, Oldendor S, Braun L, Hyman S (1972) Blood brain barrier. Penetration of morphine, codeine, heroin and methadone after carotid injection. *Science* 178: 984-986
- Paxinos G, Franklin KBJ (2001) **The mouse brain in stereotaxic coordinates**. Academics, San Diego.
- Pierce RC, Kumaresan V (2006) The mesolimbic dopamine system: The final common pathway for the reinforcing effect of drugs of abuse? *Neuroscience and Biobehavioral Reviews* 30: 215-238
- Robinson TE, Justice JBJ (1991) Microdialysis in the neurosciences. In: P. HJ (ed) **Techniques in the behavioral and neural sciences**. Elsevier, Amsterdam
- Rook EJ, Huitema AD, van den BW, Van Ree JM, Beijnen JH (2006) Pharmacokinetics and pharmacokinetic variability of heroin and its metabolites: review of the literature. *Current Clinical Pharmacology* 1: 109-118
- Schultz W (2002) Getting formal with dopamine and reward. *Neuron* 36: 241-263
- Shimomur K, Kamata O, Ueki S, Ida S, Oguri K, Yoshimur H, Tsukamot H (1971) Analgesic effect of morphine glucuronides. *Tohoku Journal of Experimental Medicine* 105: 45-52
- Skarke C, Darimont J, Schmidt H, Geisslinger G, Lotsch J (2003) Analgesic effects of morphine and morphine-6-glucuronide in a transcutaneous electrical pain model in healthy volunteers. *Clinical Pharmacology & Therapeutics* 73: 107-121
- Skarke C, Geisslinger G, Lotsch J (2005) Is morphine-3-glucuronide of therapeutic relevance? *Pain* 116: 177-180
- Smith MT (1998) Neuroexcitatory effects of morphine and hydromorphone: Evidence implicating the 3-glucuronide metabolites. *Annual Scientific Meeting of the Australasian-Society-of-Clinical-and-Experimental-Pharmacologists-and-Toxicologists.Conference Hobart, Australia*. Blackwell Science Asia. 524-528
- Sokoloff P, Giros B, Martres MP, Bouthenet ML, Schwartz JC (1990) Molecular cloning and characterization of a novel dopamine receptor (D₃) as a target for neuroleptics. *Nature* 347: 146-151
- Stein C (1993) Peripheral mechanisms of opioid analgesia. *Anesthesia and Analgesia* 76: 182-191
- Sunahara RK, Guan HC, O'Dowd BF, Seeman P, Laurier LG, Ng G, George SR, Torchia J, Van Tol HHM, Niznik HB (1991) Cloning of the gene for a human D₅ receptor with higher affinity for dopamine than D₁. *Nature* 350: 614-619

- Torregrossa MM, Kalivas PW (2008) Microdialysis and the neurochemistry of addiction. *Pharmacology Biochemistry and Behavior* 90: 261-272
- Van Tol HHM, Bunzow JR, Guan HC, Sunahara RK, Seeman P, Niznik HB, Civelli O (1991) Cloning of the gene for a human dopamine D4 receptor with high affinity for the antipsychotic clozapine. *Nature* 350: 610-614
- Venter JC, Diporzio U, Robinson DA, Shreeve SM, Lai J, Kerlavage AR, Fracek SP, Lentes KU, Fraser CM (1988) Evolution of neurotransmitter receptor systems. *Progress in Neurobiology* 30: 105-169
- Vindenes V, Handal M, Ripel A, Boix F, Morland J (2006) Conditioned place preference induced by morphine and morphine-6-glucuronide in mice. *Pharmacology Biochemistry and Behavior* 85: 292-297
- Vindenes V, Handal M, Ripel A, Thaulow CH, Vindenes HB, Boix F, Morland J (2008) Different time schedules affect conditioned place preference after morphine and morphine-6-glucuronide administration. *Pharmacology Biochemistry and Behavior* 89: 374-383
- Vindenes V, Pettersen BS, Gottås A, Lieng N, Boix F, Mørland J (Submitted-a) Different effects on dopamine release in Nucleus Accumbens in mice by the morphine metabolites M6G and M3G. *Basic & Clinical Pharmacology & Toxicology*
- Vindenes V, Ripel Å, Handal M, Boix F, Mørland J (Submitted-b) Interactions between morphine and the morphine-glucuronides measured by conditioned place preference and locomotor activity. *Pharmacology, Biochemistry and Behavior*
- Volkow ND, Fowler JS, Wang GJ, Baler R, Telang F (2009) Imaging dopamine's role in drug abuse and addiction. *Neuropharmacology* 56 (sup. 1): 3-8
- West AR, Grace AA (2002) Opposite influences of endogenous dopamine D-1 and D-2 receptor activation on activity states and electrophysiological properties of striatal neurons: Studies combining in vivo intracellular recordings and reverse microdialysis. *Journal of Neuroscience* 22: 294-304
- Wise RA (2004) Dopamine, learning and motivation. *Nature Reviews Neuroscience* 5: 483-494
- Wise RA, Rompre PP (1989) Brain dopamine and reward. *Annual Review of Psychology* 40: 191-225
- Wu DF, Kang YS, Bickel U, Pardridge WM (1997) Blood-brain barrier permeability to morphine-6-glucuronide is markedly reduced compared with morphine. *Drug Metabolism and Disposition* 25: 768-771
- Zocchi A, Girlanda E, Varnier G, Sartori I, Zanetti L, Wildish GA, Lennon M, Mugnaini M, Heidbreder CA (2003) Dopamine responsiveness to drugs of abuse: A shell-core investigation in the nucleus accumbens of the mouse. *Synapse* 50: 293-302